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TITLE: The Nuclear Death Domain Protein p84N5; a Candidate Breast Cancer Susceptibility Gene

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| 14. ABSTRACT Besides family history of cancer and an individual's age, no single etiologic factor can identify women at an increased risk for the disease. Approximately 10% of all cases of breast cancer exhibit a familial pattern of incidence. Efforts to identify the genetic basis of familial breast cancer reached fruition some years ago, when the breast-cancer susceptibility genes BRCA1 and BRCA2 were identified. However, recent studies have suggested that mutations in these genes are associated with a smaller number (20 to 60%) of hereditary breast cancer families than originally estimated, especially in studies that have been based on population-based family materials. Several groups, including ours, are searching for additional breast cancer susceptibility genes using whole genome scanning approaches, but the success of many of these approaches depends on the underlying heterogeneity of the remaining cancer susceptibility loci. The failure, to date, to identify additional breast cancer susceptibility genes associated with a high risk of disease suggests that more than one may exist. We have taken the approach that the next BRCA genes will be those that encode for proteins whose functions are linked to important cell regulatory pathways. We have recently found one such candidate BRCA3 protein, referred to as TREX84 (p84N5). | | | | | |
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INTRODUCTION:

A major challenge to breast cancer researchers has been and continues to be the ability to distinguish genetic alterations that are critical to tumor initiation from those that are epiphenomena of genetic instability. A small percentage of total breast cancer cases (~10%) are attributed to inherited mutations in highly penetrant breast cancer susceptibility genes, such as *BRCA1* and *BRCA2* [reviewed in (1)]. However, the majority of the tumors occur in women with little or no family history and the molecular basis of these sporadic breast cancers is still poorly defined. Amplification or over-expression of oncogenes (for example *c-MYC*, *ERBB2*, cyclin D1, *EGFR*, γ -synuclein) and loss of *TP53*, *PTEN* (phosphatase and tensin homolog deleted on chromosome 10), *PTCH* (patch), *MKK4* (MAP kinase kinase 4), *BRCA1*, *BRCA2*, and *HIN-1* (high in normal 1) have been shown to be present in sporadic disease (1-14). Epigenetic changes, such as inactivation of *BRCA1* due to promoter hypermethylation, have also been described in a portion of breast carcinomas (15-17). We have recently discovered that the protein, referred to as p84N5 is abnormally expressed in the vast majority of breast cancers (Guo et al., Cancer Research, 2005).

1.c Nuclear Matrix Protein, p84N5

The *p84N5* gene, located on chromosome 18p11.32, was originally isolated on the basis of its ability to encode a protein that specifically associates with the N-terminal half of p110^{RB} (18). This study demonstrated that p84N5 is a nuclear matrix protein that localizes to subnuclear regions associated with RNA processing and binds preferentially to the functionally active, hypophosphorylated form of p110^{RB}. The relevance of this interaction for RB function is not completely understood. The p84N5 protein has a region of structural similarity to the death domains of several well-characterized proteins involved in apoptosis, including tumor necrosis factor receptor 1 (TNFR-1) (19). It is thought that p84N5 functions in an apoptotic-signaling pathway initiated from within the nucleus in response to DNA damage (19-21). In addition, the p84N5 protein in cell lines has a specific subcellular nuclear localization that gives a characteristic punctate staining pattern in cells (18). Furthermore, we have found that p84N5 does not appear to be expressed in normal breast ductal epithelial cells, but is expressed in the majority of breast tumors and tumor cell lines. In a survey of the various hereditary cancer syndromes, at least four proto-oncogenes are found to be activated (i.e., *RET*, *MET*, *c-KIT*, *CDK4*) (22, 23). In general, such activation of oncogenes in the germline is embryonic lethal; however, this is not the case with these proteins. Instead, a second hit is observed in the cancer, which leads to two mutant copies (loss of heterozygosity) and/or trisomy (two mutant and one wild-type allele). We hypothesize that p84N5 may be a proto-oncogene, and when over-expressed or mutated contributes to the development of both sporadic and familial forms of breast cancer.

BODY

Progress Report Year 3 Summary

Note: The grant was extended for one year since the Postdoctoral Associate working on this project obtained an independent faculty position and left the laboratory to establish his own group, and the new Postdoctoral Associate was only recently hired.

Task 1 (Months 1-18). To evaluate the expression of p84N5 in clinical breast tumor samples and correlate with predictive factors and clinical outcomes.

We have exceeded the expectations of this first task and continue to evaluate the extent of p84N5 expression in breast cancer. Our findings were published in a manuscript in April 2005 (Guo, et al. Cancer Res. 2005, 65:3011-3016) and presented at the 96th annual AACR meeting in Anaheim, CA. We were also selected to present our studies as an oral report at the ERA of HOPE meeting in June 2005. Furthermore, we have submitted a manuscript entitled "Translational Regulation of hTREX84: A Link

Between Transcriptional Elongation, mRNA Export and Cancer,” which explores the mechanism of aberrant p84N5 expression in cancer. The paper was well-received and we are currently addressing concerns raised by the reviewers. Overall, our results indicated that p84N5 is not only a marker of breast cancer progression, but may also contribute to other forms of cancer, including ovarian. Since, over-expression of and not mutations in p84N5 were observed in breast tumors (Task 2), we investigated the mechanisms regulating gene expression. We found that aberrant methylation was not involved in regulating *p84N5* expression; however, we demonstrated that RelA/p65 might play a pivotal role. In summary, our results suggest that p84N5 could be a predictive marker of tumor progression and a potential therapeutic target for treatment of breast cancer. We have included some of the results from our published and unpublished studies.

Task 2 (Months 1-36). To evaluate *BRCA1* and *BRCA2* mutation-negative breast cancer-prone kindreds for germline *p84N5* mutations.

We have also made extensive progress addressing the goals outlined in this objective. Unfortunately, no germline or somatic mutations were found in *p84N5* leading us to explore other mechanisms regulating its expression in breast tumors (as outlined above, and shown below). Specifically, we first screened DNA isolated from blood of affected probands in *BRCA1* and *BRCA2* mutation-negative breast cancer-prone kindreds for germline *p84N5* mutations by direct DNA sequencing. We screened 45 women with breast cancer who reported at least one first degree relative with breast or ovarian cancer, and observed no deleterious mutations. Additionally, 72 cases of sporadic breast cancer and 30 cases of ovarian cancer were screened, as well as 35 cases of EBV-transformed lymphocytes, which were generated from *BRCA1* and *BRCA2* mutation-negative breast cancer-prone kindreds by Western blot analysis. We used this approach as a means to identify tumor samples potentially carrying a truncated form of the protein. However, all of the samples tested only expressed wild-type p84N5 protein. Examples of these studies are included below.

Project Results

Task 1-Progress Report–“Evaluate the expression of p84N5 in clinical breast tumor samples and correlate with predictive factors and clinical outcomes.”

A. Expression of p84N5 is also associated with human ovarian cell proliferation

As indicated above, once Task 1 was completed we decided to expand our studies. We observed that the expression of p84N5 in breast tumors was inversely related to hormone receptor status (Guo et al, 2005). Therefore, we decided to compare *p84N5* mRNA expression in 6 reduction mammoplasty specimens, including 3 nulliparous premenopausal and 3 parous premenopausal women. We observed that *p84N5* mRNA levels were substantially higher in the nulliparous specimens (data not shown). These results indicated that *p84N5* is not only deregulated in breast tumors, but also regulated during normal human breast lobular differentiation and might be modified by some hormones, such as human chorionic gonadotropin (hCG). We next asked whether *p84N5* might also be aberrantly expressed in other hormone-dependent tumors, such as ovarian tumors. As expected, p84N5 was highly expressed in all 30 cases of ovarian epithelial tumors examined (data not included). Further, we determined p84N5 protein expression (p84N5/beta-actin ratio) in primary human ovarian surface epithelial (HOSE) cell cultures (n=10), SV40 Tag immortal HOSE cell lines (n=10) and ovarian tumor cell lines (n=11) by Western blotting analysis. We found that p84N5 expression is significantly elevated in immortal cell lines (average value, 0.51) as compared to primary epithelial cells (average value, 0.125; p=0.00024) and reaches its highest level in cancer cell lines (average value, 2.10; p=0.0022) (**Figure 1a, b**).

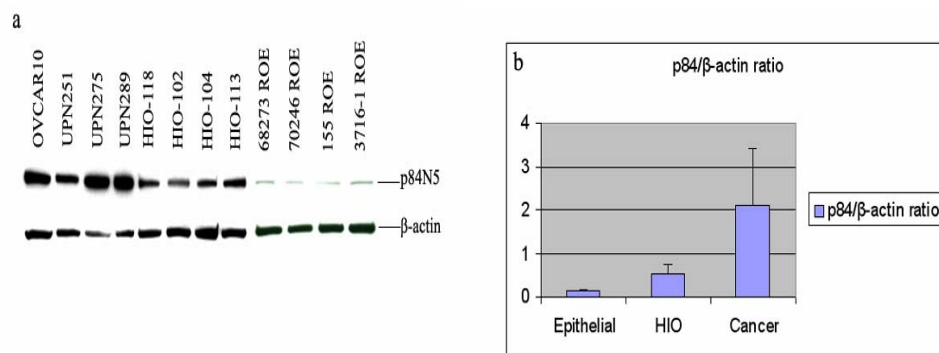


Figure 1. p84N5 is aberrantly expressed in ovarian cancer cells. (a), p84N5 protein expression in representative ovarian cancer cell lines (OVCAR10, UPN251, UPN275, UPN289), immortal epithelial cell lines (HIO-118, HIO-102, HIO-104, HIO-113), primary human surface epithelial cells (ROE). Protein samples were separated on a SDS-polyacrylamide gel and transferred proteins immunoblotted using anti-p84N5 or β-actin monoclonal antibodies. (b), p84N5/β-actin ratio in primary ovarian epithelial cell cultures (epithelial), immortal epithelial cell lines (HIO) and cancer cell lines (cancer).

To further elucidate the biological significance of *p84N5* in cancer, siRNA against *p84N5* was transfected into OVCAR10 cells. RT-PCR analysis using oligonucleotide primers specific to the *p84N5* gene showed that the expression level of the *p84N5* transcript decreases 70~80% by following transfection of *p84N5* siRNA as compared to cell transfected with control siRNA (**Figure 2a**). *p84N5*-targeted siRNAs effectively reduced the protein levels of p84N5 without affecting levels of non-targeted transcripts, such as β-actin (**Figure 2b**). Immunostaining confirmed that p84N5 protein was drastically decreased in the majority of the treated cells (**Figure 2c**). Visually, the total number of cells decreased significantly following treatment with *p84N5*-siRNAs as compared to cells treated with transfection reagent or control-siRNA (**Figure 2d**). We observed that cell growth was reduced in cells treated with *p84N5*-siRNA as compared to control (**Figure 2e**). The GuavaNexin assay showed that there was also a reduction of Annexin V-PE- and 7-AAD-positive cells upon treatment with *p84N5*-siRNAs compared to control siRNA, although the differences were not significant ($p>0.05$) (data not shown). In order to evaluate the mechanism of *p84N5* siRNA action, we further determined the cell cycle distribution by flow cytometry and found that the number of cells in G2-M phase was decreased while the number of cells in G1 phase was increased in OVCAR10s treated with *p84N5* siRNA versus control siRNA, indicating that p84N5 may be necessary for entry into the G2-M phase (**Figure 2f**). These results indicated that aberrant expression of *p84N5* may contribute to ovarian cancer, as well as breast cancer, by promoting cell proliferation.

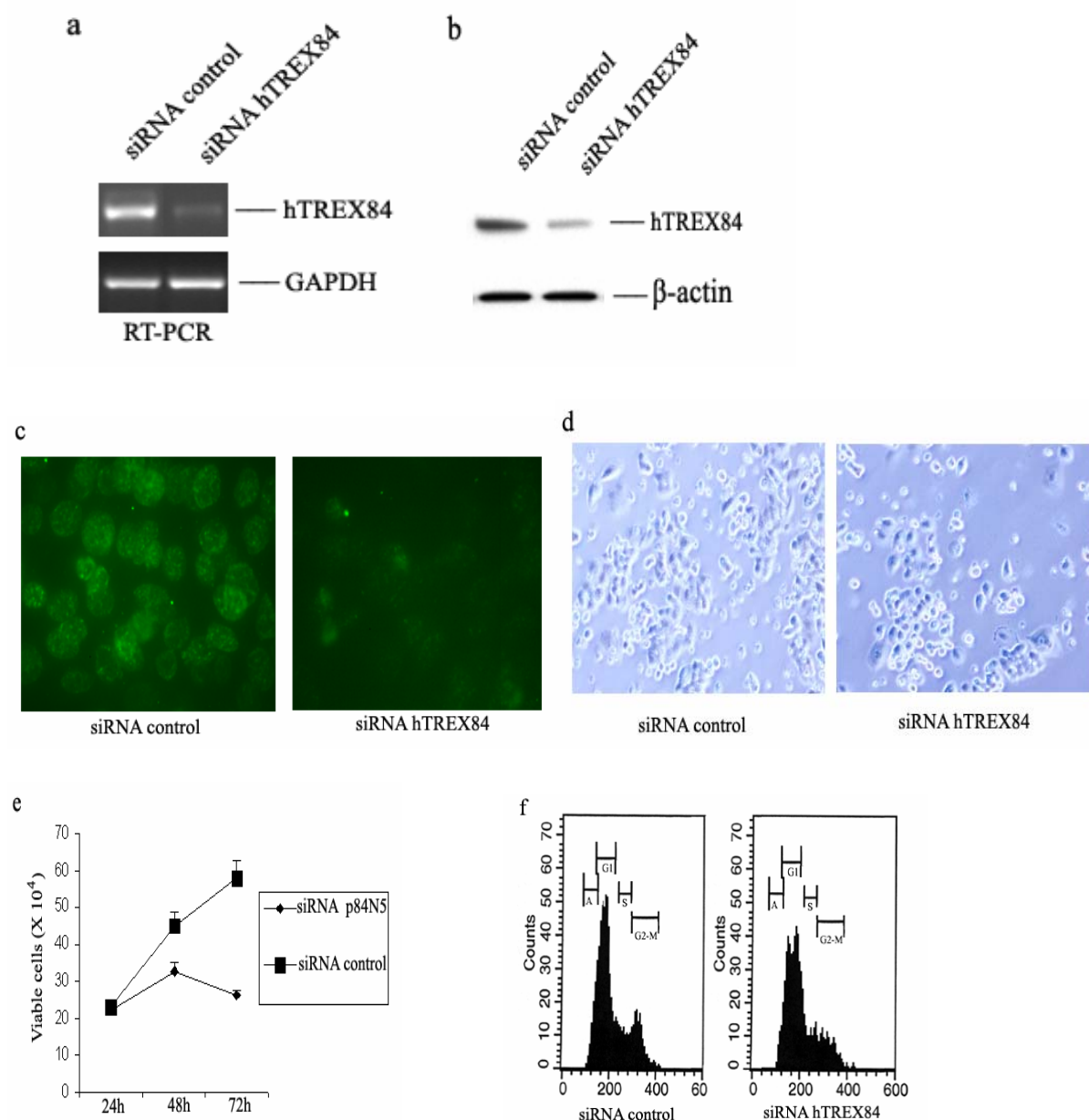


Figure 2. Knock-down of *p84N5* leads to defects in cellular proliferation of OVCAR10. (a), Analysis of *p84N5* and *GAPDH* mRNA levels following treatment of cells with siRNA against *p84N5* or control siRNA. (b), Analysis of *p84N5* and β-actin protein levels after treatment of cells with siRNA against *p84N5* or control siRNA. (c), Analysis of *p84N5* expression following siRNA treatment for 72 h by immunofluorescent staining of cells (left, cells transfected with control siRNA; right, cells treated with *p84N5*-siRNA). (d), Photomicrographs show the morphology of the cells following abrogation of *p84N5* expression (left, tumor cells transfected with control siRNA; right, cells treated with *p84N5*-siRNA). (e), Tumor cell proliferation following abrogation of *p84N5*. Cell proliferation and apoptosis (data not shown) were examined using Guava ViaCount and Nexin assays, respectively. The number of viable cells (x10⁴) at 24, 48, and 72 hrs after treatment with control or *p84N5*-siRNA is shown; data are from three independent experiments. (f), FACS analysis of the cells following down-regulation of *p84N5* levels. The comparison of cell cycle distribution after 72 h of treatment with either control siRNA (left) or *p84N5*-siRNA (right).

B. p84N5 is a subunit of the TREX complex

To gain insight into the biological role of p84N5, we isolated a p84N5-containing multiprotein complex from mammalian cells. This was accomplished by developing a 293-derived stable cell line expressing Flag-tagged p84N5. **Figure 3A** depicts the purification of Flag-p84N5 using anti-Flag antibodies followed by the analysis of the Flag-p84N5 eluate using gel filtration chromatography. This analysis revealed the specific association of p84N5 with polypeptides of 125, 120, 90, 45, 40, and 30K molecular mass (**Figure 3B and C**). Interestingly, mass spectrometric sequencing of p84N5-associated polypeptides revealed the identity of p84N5 associated proteins as the human counterparts of the yeast TREX complex reported to couple transcriptional elongation and mRNA export (**Figure 3D**). Therefore, we have termed this complex human TREX and p84N5 as hTREX84. Importantly, in contrast to the yeast TREX complex, the human complex was devoid of the RNA export and splicing factors ALY and UAP56. We therefore asked whether endogenous ALY and hTREX84 form a stable complex, which would be reflected by coelution of the two proteins via gel filtration. Analysis of HeLa nuclear extract by Superose 6 sizing fractionation showed distinct chromatographic elution profiles for hTREX84 and ALY proteins indicating that the two proteins are not stably associated (**Figure 3E**). However, consistent with a previous report (24), we observed the association of hTREX and ALY through the UAP56 protein (**Figure 3F**), and the colocalization of hTREX and ALY in breast tumor cells as determined by immunofluorescence assays (data not shown). These results indicated that whereas hTREX and ALY may not be stably associated, their interaction is promoted by the UAP56 protein.

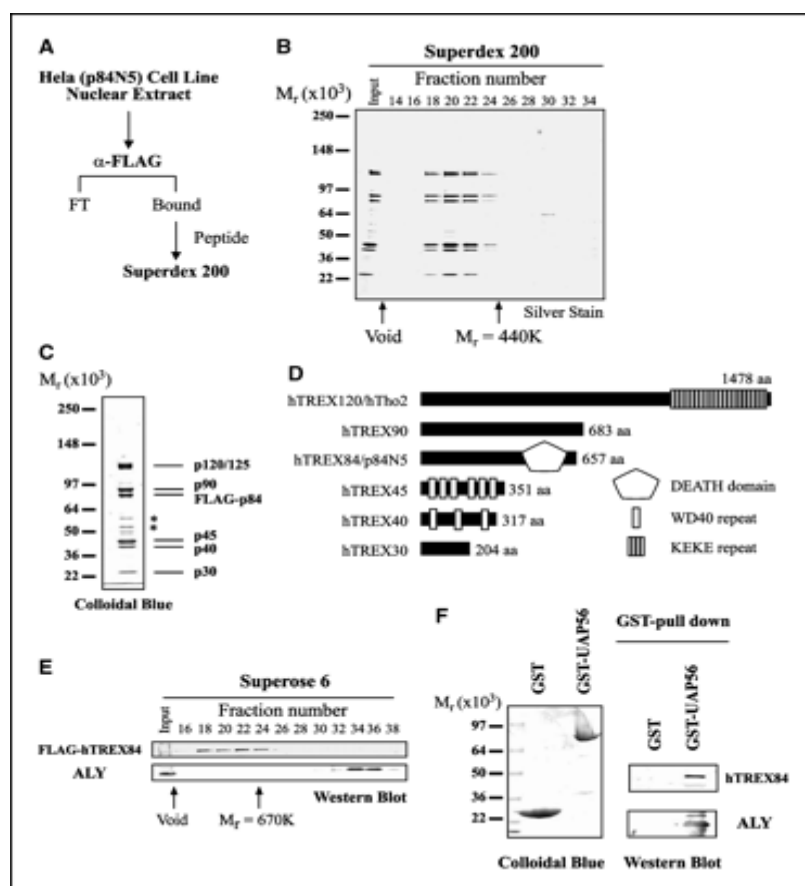


Figure 3. p84N5 is a component of the human TREX complex. **A**, Schematic of p84N5 isolation using a 293-derived Flag-tagged cell line. **B**, The human TREX complex, isolated using the protocol shown in (A), was analyzed by silver staining following fractionation on the Superset 200. **C**, Colloidal blue analysis of Flag-affinity eluate shown in (A). Individual bands were excised and subjected to mass spectrometric sequence analysis. **D**, Diagrammatic representation of human TREX subunits. hTREX120, hTREX90, hTREX45, hTREX40, and

hTREX30 correspond to Genbank accession numbers AL030996, XM_037945, NM_032361, NM_024339, and BC020599, respectively. *E*, Analysis of nuclear extract using Superose 6 gel filtration. Column fractions were analyzed by Western blotting using antibodies (*right? left*). *D*, GST or GST-UAP56 were used for affinity-purification of human TREX84 and ALY proteins.

C. Methylation status of p84N5 promoter and exon1 in cancer cells

In our previous report, we found that *p84N5* mRNA was more highly expressed in breast tumors of grade III than those of grade II by quantitative real-time PCR (qPCR). Moreover, *p84N5* mRNA was also higher in malignant epithelial cells than in normal mammary ductal epithelial cells as shown by qPCR analysis on samples obtained by laser capture microdissection (LCM). Therefore, we speculated that deregulated transcription of *p84N5* mRNA may be one of the mechanisms of p84N5 protein over-expression in cancer cells. It is well known that methylation of DNA at CpG dinucleotides has been recognized as an important mechanism for regulation of gene expression in mammalian cells (25, 26). Methylation of cytosines in the CpG sequence, located in either the promoter region or exon 1, is thought to ensure the silencing of certain tissue-specific genes in non-expressing cells. Aberrant methylation is now considered an important epigenetic alteration occurring in human cancer. Hypermethylation of normally unmethylated tumor suppressor genes correlates with a loss of expression in cancer cell lines and primary tumors. On the other hand, failure to repress genes appropriately, through abnormal demethylation of tissue-restricted genes or by hypomethylation of proto-oncogenes, could result in the loss of tissue specificity and could promote cancer formation. To elucidate the molecular mechanisms underlying the abnormal transcription of *p84N5* in carcinogenesis, a cell line expressing low levels of p84N5 were treated with a demethylating agent, 5-aza-2'-deoxycytidine (5-aza-C), at concentrations of 1, 5, 10, 50 μ M for 5 days. Total RNAs were isolated, and RT-PCR with specific primers to *p84N5* or β -actin cDNA was conducted. The results showed that the intensities of RT-PCR product of *p84N5* were increased by the 5-aza-C treatment in a dose-dependent manner. By contrast, the products of β -actin were evenly amplified from all the samples, illustrating that the expression of β -actin was not altered by the 5-aza-C treatment (**Figure 4a**). p84N5 protein was also increased in the same manner as assayed by Western blotting (**Figure 4b**). Similar results were obtained when using breast tumor cell lines, in which endogenous p84N5 was expressed at low basal levels (data not shown). Genomic DNA was subsequently isolated from these 5-aza-C treated cells and sodium bisulfite DNA sequencing was performed. Surprisingly, the results demonstrated that all the CpG dinucleotides located within *p84N5* promoter and exon1 regions from treated and untreated cells were demethylated, indicating hypomethylation was likely not the cause for increasing expression of *p84N5* mRNA and protein in these cells after 5-aza-C treatment (data not shown).

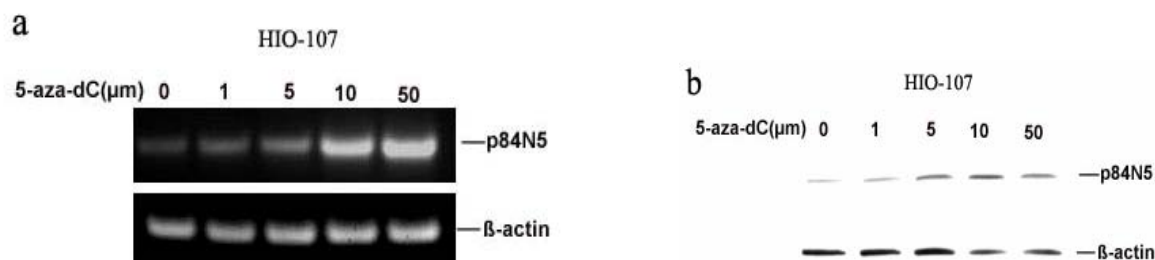
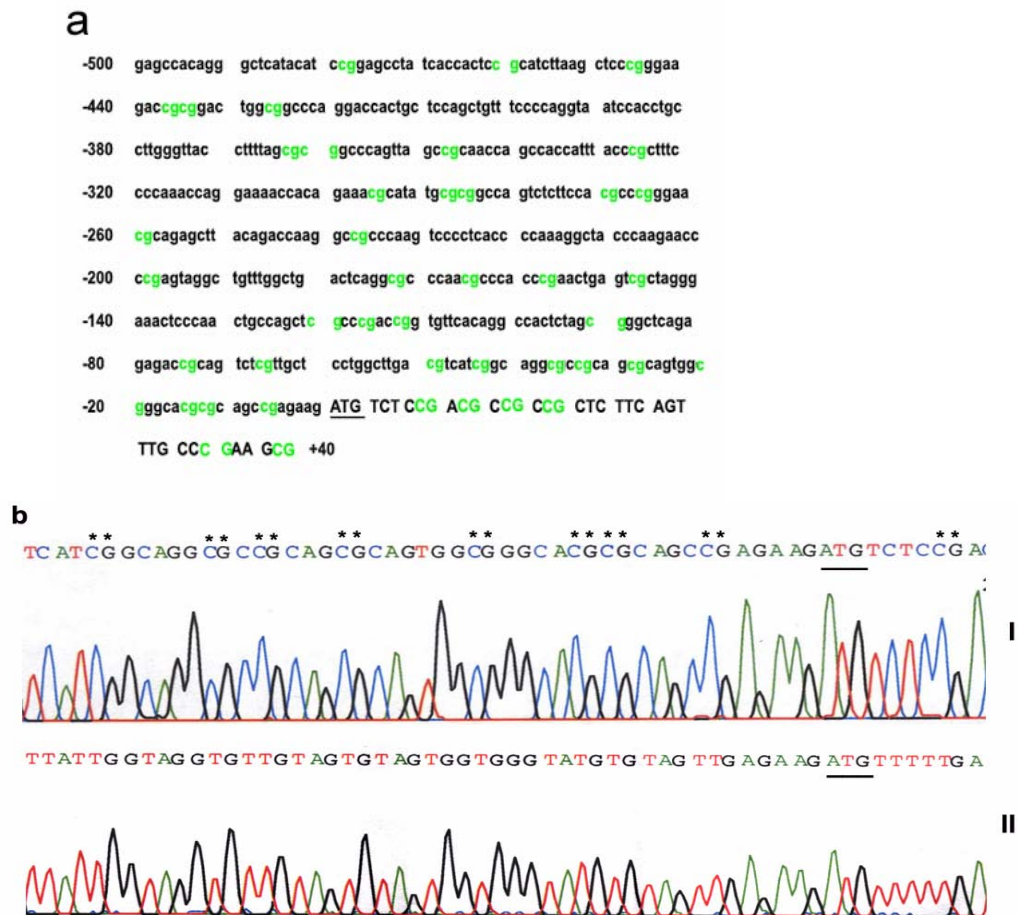


Figure 4. Methylation as a potential regulatory mechanism for *p84N5* gene expression. Cells were treated with a demethylating agent, 5-aza-C, at concentrations of 1, 5, 10, and 50 μ M for 5 days. RT-PCR (*a*), and Western blot analysis (*b*), showed *p84N5* mRNA and protein expression, respectively.

To further rule out a role for methylation in regulating *p84N5* expression, we analyzed the promoter and exon 1 regions of *p84N5* in 8 breast and 7 ovarian cancer cell lines, 10 primary mammary epithelial cell cultures, 20 cases of invasive breast ductal carcinoma, and 10 cases of ovarian tumors, as well as their paired normal tissues, by sodium bisulfite DNA sequencing. The results showed that the *p84N5* promoter and exon 1 regions in almost all cell lines were unmethylated (**Figure 5a and b**) and that the methylation status did not correlate with *p84N5* expression. The *p84N5* promoter and exon 1 regions in most normal tissues were also unmethylated, although we observed occasional methylation of a few CpG dinucleotides (**Figure 5c**). Overall, our results suggested that aberrant methylation of *p84N5* is not likely to contribute to its abnormal expression in the majority of breast tumors. There are several possibilities, which could explain why 5-aza-C can induce *p84N5* expression independently of promoter methylation. For example, 5-aza-C may have dramatic effects on chromosomes, leading to decondensation of chromatin structure, thus enhancing specific gene expression (27). Another possibility is that 5-aza-C might lead to activation of transcription factors or repression of inhibitors that regulate *p84N5* expression. Although not yet resolved, we have observed that NF- κ B is upregulated in many breast tumors and that *p84N5* contains consensus NF- κ B binding motifs in its promoter (see below).



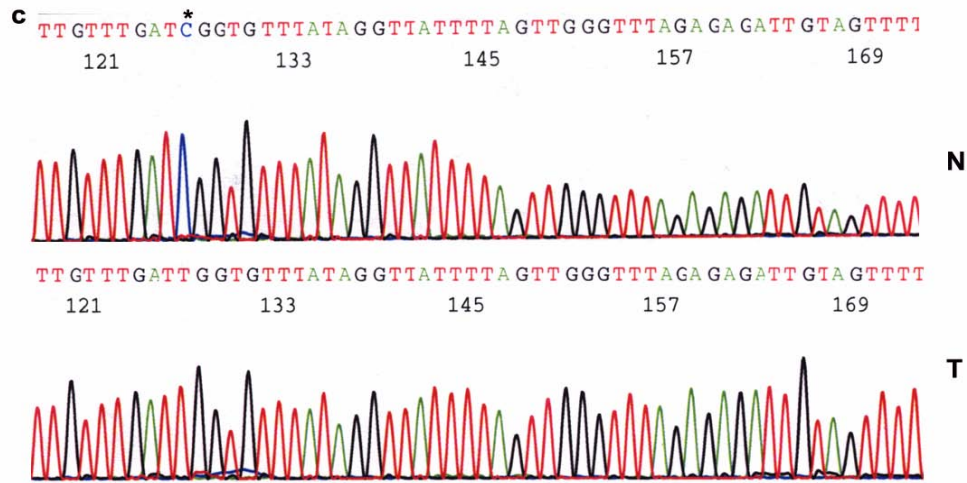


Figure 5. (a). p84N5 promoter and exon 1 region, the nucleotides in green color are CpG sites. Nucleotides are numbered on the right from the ATG translation start codon which is underlined. (b). sodium bisulfite DNA sequencing, from untreated (I) and treated DNA samples (II). Star marker indicates CpG sites. (c). Sodium bisulfite DNA sequencing, from a normal breast tissue (N) and a carcinoma tumor (T). Star marker indicates CpG sites.

D. NF- κ B activation enhances p84N5 expression in immortal and/or cancer cells

To provide a better understanding of the molecular basis of p84N5 over-expression in cell immortalization and carcinogenesis, we took the approach to identify the transcription factor binding sites in the *p84N5* promoter (32) using the AliBaba2 tool, available at <http://www.witi.cs.uni-magdeburg.de/grabe/alibaba2>. 9-SP1, 7-NF1, 4-AP1, 2-NF- κ B, together with other transcriptional factor consensus binding sequences, were found in the *p84N5* promoter by this program. We focused on and validated nuclear factor of κ B NF κ B for several reasons. NF κ B is not a single protein, but a small menagerie of closely related protein dimers that bind a common sequence motif known as the κ B site (33). According to Hanahan and Weinberg, tumorigenesis requires six essential alterations to normal cell physiology: self-sufficiency in growth signals; insensitivity to growth inhibition; evasion of apoptosis; immortalization; sustained angiogenesis; and tissue invasion and metastasis (34). NF- κ B is able to induce several of these cellular alterations (35), and has been shown to be constitutively activated in some types of cancer cells, including breast cancer. Previous studies have documented elevated or constitutive NF- κ B DNA-binding activity both in mammary carcinoma cell lines and primary breast cancer cells of human and rodent origin (36-38). This could be correlated with the increased level of epithelial growth factor family receptors (EGFR) (39). The chromatin immunoprecipitation (ChIP) assay is a powerful technique to determine true *in vivo* binding of transcription factors and other nucleosomal proteins to chromatin (40, 41). We used this assay to determine status of RelA/p65, one subunit of NF- κ B, at the promoter of *p84N5*. After the ChIP protocol, p84N5 gene promoter regions were amplified and analyzed by semiquantitative PCR using specific primer pairs around NF- κ B binding regions on the promoter of *p84N5* (**Figure 6a**). MDA-MB-231, OVCAR10, OVCAR5 cells cultured for 3 days were subjected to ChIP with and without antibody to p65. Enrichment of specific DNA sequences in the chromatin immunoprecipitates, indicating association of p65 to DNA strands within intact chromatin, were visualized by PCR amplification. No binding was seen for immunoprecipitated samples without p65 antibody (**Figure 6b**). These results were further confirmed when we transiently transfected p65 expression plasmid into MCF-10F cells and p84N5 protein was also increased (**Figure 6c**). Moreover, when we knocked down p65 expression by siRNA targeted p65, p84N5 protein also decreased, as

predicted (**Figure 6d**). In summary, these results show for the first time that RelA/p53 plays a pivotal role in regulating the p84N5 expression.

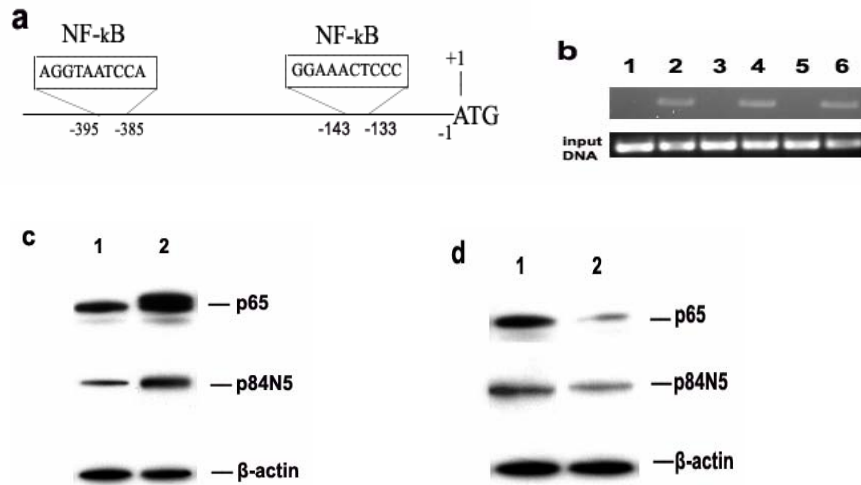


Figure 6. The role of NF- κ B in regulation p84N5 expression. (a), Schematic diagram of the *p84N5* promoter, indicating the NF- κ B DNA binding motif. (b), ChIP assays of p53 binding to p84N5 gene promoter in MDA-MB-231 (lanes 1, 2); OVCAR5 (lanes 3, 4); OVCAR 10 (lanes 5, 6). Cells were cultured for 72 h. ChIP assays were then performed with anti-p53 antibody. PCR analysis was performed on immunoprecipitation samples without antibody (lane 1, 3, 5), with p53 antibody (lane 2, 4, 6). (c), Western blot analysis of p53, p84N5 and β -actin after MCF-10F cells were transiently transfected with control vector (lane 1), or with p53 plasmid (lane 2), for 48 h. (d), Western blot analysis of p53, p84N5 and β -actin protein levels after treatment of MDA-MB-231 cells with control siRNA (lane 1) or siRNA against p53 (lane 2) for 72 h.

Table 1. RelA/p53 expression in human normal breast tissue and tumors

| | N | 0/+ | ++ | +++ |
|-------------------------------|----|-----|----|-----|
| Normal breast tissue | 5 | 4 | | 1 |
| Tumor histologic grade | | | | |
| 1 (well differentiated) | 22 | 11 | 7 | 4 |
| 2 (moderately differentiated) | 33 | 7 | 16 | 10 |
| 3 (poorly differentiated) | 34 | 3 | 10 | 21 |

Since our previous studies demonstrated that hTREX84 was highly expressed in the cell nucleus,

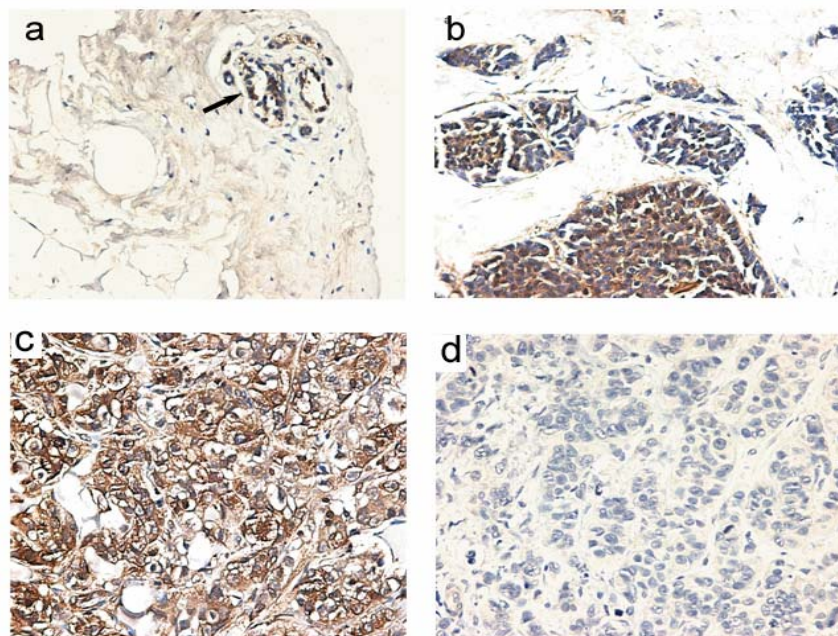


Figure 7. Immunohistochemical staining of RelA/p65 proteins in representative breast cancer tissue specimens. *A*, RelA/p65 was weakly detected in normal breast epithelial cells and positive products were located in the cell cytoplasm. *B*, Staining for RelA/p65 was detected mainly in the cytoplasm in high differentiated tumors. *C*, Intense and distinctly granular staining for RelA/p65 was detected in stained nuclei of low differentiated tumor specimens. *D*, Tumor section evaluated without the primary antibody to serve as a negative control. Magnification 200x.

especially in poorly differentiated and more aggressive human breast cancers, we asked whether RelA/p65 might also be expressed in a similar manner. We examined the protein expression of RelA/p65 by immunohistochemical analysis in 89 cases of human breast cancer, as well as 5 normal breast tissues (**Table 1**). This tumor panel includes 22, 33 and 34 cases of well, moderately and poorly differentiated tumors, respectively. RelA/p65 was weakly (0/+1) detected in normal breast epithelial cells (4 of 5) and protein staining indicated cytoplasmic localization (**Figure 7a**). Staining for RelA/p65 was also observed mainly in the cytoplasm in well-differentiated tumors (**Figure 7b**). Distinctly granular staining with an increased number of positively stained nuclei was observed in the poorly differentiated tumor specimens (+2/+3, 31 of 34) (**Figure 7c**). Similar expression patterns of RelA/p65 and hTREX84 in normal and

malignant cells suggests a correlation between expression and tumor progression and metastasis.

Additional findings relevant to p84N5's role in the pathogenesis of breast cancer.

In our previous report, we observed that p84N5 protein is undetectable in most normal breast tissues by Western blot analysis. However, *p84N5* mRNA is detected by RT-PCR analysis. We speculated that a mechanism of p84N5 protein level regulation, which is independent of RNA level regulation, might exist in the normal cell. Since we lacked appropriate cultured normal breast epithelial cells for this study, we selected a model of 32D cells, which are well-characterized diploid murine hemopoietic cells. 32D cells have an absolute requirement for interleukin-3 (IL-3), and undergo apoptosis when IL-3 is withdrawn. When we induced the cell to differentiate by Granulocytic-Colony Stimulating Factor (G-CSF), p84N5 protein rapidly disappeared on the second day; however mRNA levels decreased but were still present even after 5 days. When we treated cells with Lactacystin, a proteasome inhibitor, p84N5 degradation was significantly delayed. Further, p84N5 protein was not able to bind ubiquitin, indicating p84N5 protein degradation during cell differentiation might occur through a ubiquitin-independent pathway.

Task 2 – Progress Report “To evaluate *BRCA1* and *BRCA2* mutation-negative breast cancer-prone kindreds for germline *p84N5* mutations.”

We designed four sets of primers specific for *p84N5* cDNA and performed DNA sequence analysis (**Table 2**). The results showed that all cancer cell lines have several nuclear base changes that are different from the original cloned *p84N5* cDNA sequence (GenBank NM_005131), but identical to the later predicted *p84N5* cDNA sequence (GenBank XM_008756), by automated computational analysis using a gene prediction method, BLAST, supported by mRNA and EST evidence as well as our cDNA sequence (**Table 3**). To further confirm this, we sequenced cDNA from 7 SV40 Tag immortal HOSE cell lines (HIO-102, HIO-113, HIO-114, HIO-117, HIO-121, HIO-135, HIO-166), which were derived and maintained in our laboratory, as well as cDNA from 6 ovarian cancer cell lines (OVCAR2, OVCAR3, OVCAR4, UPN251, UPN275 and A2780). All the ovarian cell lines showed cDNA sequence results identical to those of breast cancer cell lines. Based on these data, we concluded that there are no somatic mutations in *p84N5* in these cell lines.

We also tested 45 cases of *BRCA1* and *BRCA2* mutation-negative breast cancer-prone kindreds for germline *p84N5* mutations by EMD and direct DNA sequencing. Again, no germline mutations were detected. Interestingly, we failed to observe polymorphisms in *p84N5* sequence. Additionally, 72 cases of sporadic breast cancer and 30 cases of ovarian cancer, as well as 35 cases of EBV-transformed lymphocytes, which were generated from *BRCA1* and *BRCA2* mutation-negative breast cancer-prone kindreds, were screened by Western blot analysis. No aberrant protein bands were observed (data not shown). Therefore, activation of *p84N5* is independent of mutations and is associated with overexpression.

Table 2. Oligonucleotide primer pairs for PCR amplification of the entire cDNA sequence of *p84N5*

| Primer pairs ¹ | Oligonucleotide sequences (5'-3') |
|-------------------------------------|--|
| 47U/515L | 5'-CTCTTCAGTTTGCCCGAAGC/AAAAGAGCTGAATCCGTCCA-3' |
| Exons 1-7, fragment length 469 bp | |
| 506U/1033L | 5'-CAGTCTTCTGTGGACGGATTC/ATGCCCCTTGAGATATTGGA-3' |
| Exons 7-12, fragment length 548 bp | |
| 1026U/1576L | 5'-CAAGTGAAAAGCTGATGGATTT/TTTAAACTGCTGGTTGGTTGG-3' |
| Exons 11-19, fragment length 641 bp | |
| 1442U/2083L | 5'-GAACAGGCAGACCCTGAAAA/CCAAAACCAGTGGACCTCTT-3' |
| Exons 18-21, fragment length 642 bp | |

¹ Primers were designated by nucleotide position to *p84N5* (GenBank XM_00876) corresponding to the 5' position, followed by the letter U for upper (*i.e.*, sense strand) or L for lower (*i.e.*, anti-sense strand).

Table 3. The nuclear base and corresponding amino acid differences of cDNA in the cell lines from the original *p84N5* cDNA sequence

| Exon | Nucleotide position-database (encoded amide acid) | Nucleotide position-cell lines (encoded amino acid) |
|------|--|--|
| 6 | 388G (A) | 388T (S) |
| 9 | 657T | 657C |
| 16 | 1298C (T) | 1298T (M) |
| 18 | 1438C (A) | 1438T (V) |
| 19 | 1458A (M) | 1458G (V) |
| 19 | 1493A (K) | 1493G (R) |
| 19 | 1556A (Q) | 1556C (P) |

KEY RESEARCH ACCOMPLISHMENTS (5/2004 to present):

1. “The nuclear death domain protein p84N5; a candidate breast cancer susceptibility gene”

- 1.a. Demonstrated that TREX84 (p84N5) is expressed at very low levels in normal breast epithelial cells and that it is highly expressed in breast tumors.
- 1.b. Report that TREX84 expression correlates with tumor size and the metastatic state of the tumor progression, i.e., identify TREX84 as a prognostic marker for aggressive forms of human breast cancer.
- 1.c. Identified that TREX84 is the human counter part of the yeast TREX complex reported to couple transcriptional elongation and mRNA export.
- 1.d. Demonstrated that TREX84 is over-expressed in both breast and ovarian tumors.
- 1.e. Abrogation of *TREX84* expression leads to growth arrest in both breast and ovarian tumor cell lines.
- 1.f. Found that 5-aza-C can induce TREX84 expression that is not dependent on p84N5 promoter methylation.
- 1.g. Identified several transcriptional factors, including NF- κ B binding sites in the *hTREX84* gene promoter and demonstrated by chromatin immunoprecipitation (ChIP) and site directed mutagenesis that RelA/p65 binds the NF- κ B binding sites and stimulates *hTREX84* expression.
- 1.h. Demonstrated by immunohistochemistry (IHC) that RelA/p65 is abundantly expressed in malignant cells that aberrantly express hTREX84 indicating that RelA/p65 might play a pivotal role in regulating hTREX84 expression in breast cancer.

REPORTABLE OUTCOMES (5/2004 to present):

I. “The nuclear death domain protein p84N5; a candidate breast cancer susceptibility gene”

1.a. Abstracts

Shan-Chun Guo and A.K. Godwin. Accumulation of p84N5 domain protein is associated with an aggressive phenotype of human breast tumors. Proceedings of American Association of Cancer Research, 44:2421, 2003.

Guo, S., Farber, M.J., Shiekhatter, R. and Godwin, A.K. Over-expression of death domain containing protein-p84N5 in human ovarian cancer cell lines is associated with cell proliferation. Proceedings of American Association of Cancer Research, 44:1805, 2004.

Guo, S., Hakimi, M-A., Baillat, D., Chen, X., Farber, M.J., Klein-Szanto, A.J.P., Cooch, N.S., Shiekhatter, R., Godwin, A.K. Coupling transcriptional elongation and mRNA export to metastatic breast cancers. Proceedings of American Association of Cancer Research, 46:5588, 2005.

Guo, S., Hakimi, M-A., Baillat, D., Chen, X., Farber, M.J., Klein-Szanto, A.J.P., Cooch, N.S., Shiekhatter, R., Godwin, A.K. Linking transcriptional elongation and mRNA export to metastatic breast cancers. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, June 8-11, 2005, P40-6 (selected for oral presentation)

1.b. Publications

Guo, S., Hakimi, M-A., Baillat, D., Chen, X., Farber, M.J., Klein-Szanto, A.J.P., Cooch, N.S., Shiekhatter, R., Godwin, A.K. Linking Transcriptional Elongation and mRNA Export to Metastatic Breast Cancers. Cancer Res. 65(8):3011-3016, 2005.

Guo, S., Vanderveer, L., Farber, M.J. Godwin, A.K. Translational Regulation of hTREX84: A Link Between Transcriptional Elongation, mRNA Export and Cancer. Submitted, 2006.

Book chapters and review articles:

Pan, Z-Z., Godwin, A.K. Oncogenes. Encyclopedia of Molecular Cell Biology and Molecular Medicine. Edited by R.A. Meyers, Second Edition, Volume 9. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, pp., 435-495, 2005.

Bui, C.T., Nicolas, E., Sallmann, G., Chiotis, M., Lambrinakos, A., Rees, K., Trounce, I., Cotton, R.G.H., Hancock, L., Godwin, A.K., Yeung, A.T. Enzymatic and Chemical Cleavage Methods to Identify Genetic Variation. In Molecular Diagnostics (Ed. G. Patrinos and W Ansorge) in press 2006.

Chen, X., Arciero, C., Godwin, A.K. BRCA1-associated complexes: New targets to overcome breast cancer radiation resistance. Expert Review of Anticancer Therapy, 6:187-196, 2006.

CONCLUSIONS:**1. “The nuclear death domain protein p84N5; a candidate breast cancer susceptibility gene”**

THO/TREX is a conserved eukaryotic complex containing Tho2, HPR1, MFT1 and Thp2, as well as proteins involved in mRNA metabolism and export such as Aly and UAP56. In the present work, we identified human p84N5 (referred to as hTREX84) as a conserved counterpart of yeast protein HPR1 (Tho1) and for the first time, demonstrated that aberrant hTREX84 expression is associated with not only human breast cancer, but also ovarian cancers. We report that the demethylation agent, 5-aza-2-deoxycytidine, significantly induced p84N5 mRNA and protein expression, suggesting that abnormal expression of p84N5 might be mediated by epigenetic mechanisms. We also have found that RelA/p65 plays a pivotal role in regulating the p84N5 expression. Other mechanisms such as protein abnormal degradation might also contribute p84N5 over-expression in cancer cells. Thus p84N5 might be served as good tumor proliferation marker as well as an ideal target for therapeutic drugs against cancer.

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